Sphingosine Kinase 1 Is Up-regulated during Hypoxia in U87MG Glioma Cells

ROLE OF HYPOXIA-INDUCIBLE FACTORS 1 AND 2

Viviana Anelli†§, Christopher R. Gault‡, Amy B. Cheng¶, and Lina M. Obeid†‡¶

From the†Ralph H. Johnson Veterans Affairs Medical Center, Charleston, South Carolina 29401, the Departments of‡Medicine and¶Biochemistry and Molecular Biology, Medical University of South Carolina, Charleston, South Carolina 29403, and the§Department of Medical Chemistry, Biochemistry, and Biotechnology, University of Milan, Segrate, Milan 20090, Italy

Sphingosine 1-phosphate (S1P), sphingosine 1-phosphate, structural backbone of all sphingolipids, is a bioactive lipid that regulates different biological processes, such as cell growth, differentiation, survival, and motility (1). Moreover, a growing body of recent evidence has implicated S1P as one of the most potent proangiogenic agents (2–4), enhancing tube formation and migration of endothelial cells (4). S1P is a peculiar molecule acting both inside the cell on as yet undefined targets and as an extracellular mediator through binding to one of five cell surface G-protein-coupled receptors of the EDG family, recently renamed S1P1–5 receptors (5). Each S1P receptor subtype activates a unique set of G proteins with varying preferences (6, 7).

S1P is produced by the action of sphingosine kinases. In mammals, two isoforms of this enzyme have been identified and cloned, namely sphingosine kinase 1 (SK1) (8, 9) and sphingosine kinase 2 (SK2) (10). These two proteins differ in their catalytic properties, cellular localization, and functions (11); SK1 is mainly cytosolic and mediates prosurvival functions (12–15), whereas SK2 is predominantly in the nucleus, where it inhibits growth and enhances apoptosis (16–18). SK1 has been shown to be activated by numerous external stimuli, such as tumor necrosis factor-α (19, 20), platelet-derived growth factor (21), VEGF (22), neural growth factor, basic fibroblast growth factor (23), EGF (24), and phorbol 12-myristate 13-acetate (25), resulting in increased intracellular S1P and increased release of S1P from certain cell types (25–28).

SK1 has been shown to act as an oncogene, whereby its overexpression regulates cell growth in soft agar and leads to tumor formation in xenograft model (29). Moreover, knockdown of SK1 has been shown to activate apoptotic pathways of cell death (15). Importantly, SK1 mRNA was found to be significantly higher in various tumor tissues, such as brain, breast, colon, lung, ovary, stomach, uterus, kidney, rectum, and small intestine. The elevated SK1 mRNA expression was confirmed by immunopositive staining for SK1 in lung and colon cancer (30, 31).

Glioblastoma multiforme is the most commonly occurring primary brain tumor in adults and displays aggressive growth...
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and invasion into surrounding brain tissue, leading to a short life expectancy after the initial diagnosis. One of the key histopathological features of gliomas is an intense increased angiogenesis attributed to increased VEGF expression (32). S1P has been shown to enhance growth, migration, and invasiveness of glioblastoma multiforme cell lines (33). Moreover, expression of SK1 in glioblastoma multiforme tissue has been shown to correlate with short patient survival (34). Gliomas, similar to other malignant tumors, are characterized by extensive regions of low oxygen tension caused by rapid cell proliferation, leading to tissue hypoxia (35).

Central to the adaptive response that occurs in tumor hypoxia is a dramatic increase of protein levels of hypoxia-inducible factors (HIFs), which are key transcriptional regulators involved in the induction of a set of hypoxia-regulated genes. HIF is composed of an oxygen-regulated HIF-α subunit (HIF-1α and HIF-2α) and the ubiquitous aryl hydrocarbon receptor nuclear translocator (or HIF-1β) partner protein (36). HIF-α protein turnover in normoxia is very rapid due to the action of HIF-α prolyl hydroxylases. These oxygen-dependent enzymes hydroxylate two conserved proline residues of HIF-α proteins, promoting binding of the Von Hippel-Lindau protein, ubiquitination, and subsequent proteosomal degradation (37). Under hypoxic conditions, the hydroxylases are inhibited, and HIF-α proteins are stabilized and transcriptionally activated, leading to potent induction of target genes. An important question in HIF biology is what roles the two HIF-α subunits exert on target gene activation, particularly the extent to which they cooperate, overlap, or have distinct roles. Although they exhibit high sequence homology and seem to be regulated in a similar fashion, it has been demonstrated that there is little redundancy between the two α subunits, and they have distinct expression and activation of target genes. For example, it has been shown that HIF-1α exclusively induces the hypoxic transcription of glycolytic genes, such as phosphoglycerate kinase 1 (pgk1) and aldolase A (aldA) (38, 39), whereas HIF-2α induces VEGF and transforming growth factor α expression (38, 40, 41).

It was recently shown that hypoxic stress induced S1P formation in vascular smooth muscle cells (42). Moreover, acute hypoxia increased SK1 and SK2 mRNA transcript levels in human pulmonary smooth muscle cells (43). In addition, cardiac myocytes null for the SK1 gene were shown to be more vulnerable to hypoxic stress (44). However, little is known about the role and regulation of SK during tumor-induced hypoxia or about SK regulation by HIFs.

In this study, we show for the first time that SK1 protein, message, and SK activity are increased in tumor cell hypoxia and in response to CoCl2, a hypoxia-mimicking agent (45). This increase in SK1 is accompanied by an increase in cellular and released S1P. We also show that HIF-2α but not HIF-1α is required for the hypoxia-induced increases in SK1 and S1P. Moreover, we demonstrate that HIF-2α binds the SK1 promoter and that this binding is increased by hypoxia. In addition, we demonstrate that conditioned media from hypoxia-treated tumor cells results in neoangiogenesis in human umbilical vein endothelial cells in a S1P receptor-dependent manner.

EXPERIMENTAL PROCEDURES

Materials—Tissue culture medium, heat-inactivated fetal bovine serum, penicillin/streptomycin, phosphate-buffered saline, oligofectamine, and Lipofectamine 2000 were from Invitrogen. Human glioma cells U87 and 786-O were from the American Type Culture Collection. HUVECs were from Cambrex. CoCl2, KCl, Tris-HCl, EDTA, deoxypyridoxine, sodium orthovanadate, β-glycerophosphate, phenylmethylsulfonyl fluoride, glycerol, Triton X-100, and ATP were from Sigma. D-erythro Sphingosine and D-erythro-sphingosine 1-phosphate were from Biomol. D-erythro-[3-3H]Sphingosine and [γ-32P]ATP were from PerkinElmer Life Sciences. Rabbit polyclonal anti-HIF-1α and HIF-2α were from Novus Biological. Rabbit anti-HSK1 was prepared by the Medical University of South Carolina antibody facility as previously described (39).

Cell Cultures—U87 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum and 100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml amphotericin B in a 5% CO2 incubator at 37 °C. 786-O cells were cultured under similar conditions in RPMI medium. HUVECs were cultured in endothelial cell medium-2 (EGM™-2) supplemented with 2% FCS and singleQuot®. For in vitro angiogenesis experiments, HUVECs were used between passages 2 and 8.

Treatment of Cells with CoCl2 and Hypoxia—Cells at 70–80% confluence were serum-starved for 16 h in DMEM or RPMI containing 0.1% fatty acid-free BSA and incubated in the absence or presence of 150 µM CoCl2. For the hypoxia experiments, cells were incubated for different times in a 5% CO2, 95% nitrogen incubator with an oxygen sensor set at 0.5% O2.

Chromatin Immunoprecipitation (ChIP) Analysis—The ChIP assay was performed according to the protocol provided with the ChIP assay kit (Upstate Biotechnology, Inc.). Briefly, U87 and 786-O cells at 70–80% confluence were serum-starved for 16 h in DMEM or RPMI containing 0.1% fatty acid-free BSA and incubated in the absence or presence of 150 µM CoCl2 for 2 h. At the end of incubation, formaldehyde was added to the plate at 1% final concentration, and cells were incubated for 10 min at room temperature. Cells were washed twice with cold phosphate-buffered saline, lysed in 1 ml of SDS lysis buffer, and sonicated three times for 10 min to shear DNA to an average fragment size of 200–1000 bp. Ten µl of each sonicated sample of cell lysate were removed and used as input control in the final PCRs. The supernatant was recovered, diluted, and precleared using Protein A-agarose/salmon sperm DNA. The recovered supernatant was incubated with either rabbit anti-HIF-1α, anti-HIF-2α, or an isotype control IgG overnight in the presence of Protein A-agarose/salmon sperm DNA. The beads were washed with low salt, high salt, and LiCl buffers. The immunoprecipitated DNA was eluted from the beads with 0.2% SDS and 0.1 × NaHCO3 solution. Eluates were then incubated with 0.2 × NaCl for 4 h at 65 °C. DNA was then purified using a PCR purification kit (Qiagen), and PCR was done on the extracted DNA using Advantage-GC Genomic PCR (Clontech) with the following specific primers for different regions of SK1 promoter: A, 5’-gtggagaggggttctca-3’ (forward) and 5’-ctcttctcagccttttctc-3’ (reverse); B, 5’-tgacag-
gtggcctttac-3’ (forward) and 5’-ctccgagaacagaggagc-3’ (reverse); C, 5’-tcgtcctgtttctcgagt-3’ (forward) and 5’-ggaga-
ggagtctagctg-3’ (reverse); D, 5’-ggtctccgagaagacag-3’ (forward) and 5’-gattgagaacgaagactg-3’ (reverse).

siRNA Down-regulation of HIF-1α, HIF-2α, and SIP1—Expression—HIF-1α was down-regulated using 50 nm sequence-specific siRNA from Invitrogen (Validated Stealth 46-3242; duplexes 1 and 2 showed similar knockdown results (see supplemental Fig. 2) and duplex 1 was used in all experiments). HIF-2α was down-regulated using 50 nm sequence-specific siRNA from Ambion (predesigned siRNA ID 106447 and 106448; both showed similar knockdown effectiveness (see supplemental Fig. 2), and the former was used in all experiments). SIP1 was down-regulated using 10 nm sequence-specific siRNA from Santa Cruz Biotechnology (catalog number sc37086). Scrambled siRNA was synthesized from Xeragon (5’-AATTCTCCGAACGTGTCAGTG-3’) and was used as a negative control. Cells were seeded in 60-mm dishes at a density of 2 × 10^5 24 h before down-regulation and transfected using Oligofectamine reagent according to the manufacturer’s protocol. Briefly, 5 μl of 20 μM siRNA (for HIF-1α and HIF-2α) or 2 μl of 10 μM (for SIP1) was resuspended in 400 μl of Opti-MEM medium and mixed with 30 μl of oligofectamine-opti-MEM medium complex (4:11, v/v). The mixture was then incubated for 20 min at room temperature and added to the cells (incubated for 20 min in 1.6 ml of Opti-MEM medium). After 4 h, 1 ml of 30% fetal bovine serum medium (DMEM or RPMI or EGMTM-2) was added to the dishes, and the cells were further incubated for 48 h. The efficiency of the knockdown was determined by quantitative real time PCR for HIF-1α, HIF-2α, and SIP1 mRNA and immunoblotting using specific antibodies 48 h after transfection.

Measurement of Promoter Activity—The hSK1 promoter was cloned from human genomic DNA through PCR amplification of a 3-kb DNA segment upstream of the SK1 5’-untranslated region. This fragment was gel-purified and cloned into TOPO-XL vector (Invitrogen) and verified by sequencing. The putative SK1 promoter was excised using KpnI and NheI and ligated into the pGL3 Basic luciferase vector.

U87MG cells were seeded at 2 × 10^5 cells in 60-mm dishes for 24 h and treated with scrambled, HIF-1α, or HIF-2α siRNA for 24 h. Cells were then co-transfected using Lipofectamine 2000 with 0.8 μg of SK1 promoter construct and 0.2 μg of β-galactosidase plasmid for 24 h. Cells were serum-starved for 16 h and treated with CoCl2 for 2 h. Cells were then lysed, and luciferase and β-galactosidase measurements were made using a luciferase and β-galactosidase enzyme assay system kit (from Stratagene) according to the manufacturer’s instructions. Data are normalized with β-galactosidase activity in each sample.

Quantitative Real Time PCR—Total RNA was isolated using the RNeasy minikit (Qiagen), treated with the Turbo RNase-free kit (Ambion), and measured using the Quant-it RiboGreen RNA kit (Invitrogen). RNA (1 μg) was reverse transcribed to cDNA using the SuperScript First-Strand Synthesis System (Invitrogen). Quantitative real-time PCR was performed with an iCycler Q real time detection system using the SYBR Green Supermix kit (Bio-Rad). Reactions were performed using hSK1-specific primers (forward primer, 5’-CTTGAACCAT-3’; reverse primer, 5’-TGTGCAGAGACAGAGGTCA-3’), hSK2-specific primers (forward primer, 5’-CCAGTGTTGGAGAGCTGAAGT-3’; reverse primer, 5’-GTCCATTCATCTCGTGGTCTC-3’), hHIF-1α-specific primers (forward primer, 5’-GAAAGCCGAAGCTCCCCT-3’; reverse primer, 5’-TGGAGTTCCTGATTGACCA-3’), and β-actin-specific primer (forward primer, 5’-ATGGCAATGACCGTGTC-3’; reverse primer, 5’-GGTAGTTTCTGATGACCA-3’). Real time PCR conditions were as follows: 3 min at 95 °C followed by 40 cycles with 45 s at 60 °C, 1 min at 95 °C, 1 min at 60 °C, and 10 min at 4 °C. Real time PCR results were analyzed using Q-Gene® software, which expresses data as mean normalized expression. All of the genes were normalized to expression of β-actin as an endogenous control.

Sphingosine Kinase Activity—Sphingosine kinase activity was determined as described previously with minor modifications (46). After CoCl2 treatment cells were washed with cold phosphate-buffered saline and harvested in SK1 buffer (containing 20 mm Tris-HCl, pH 7.4, 1 mm EDTA, 0.5 mm deoxypyridoxine, 15 mm NaF, 1 mm β-mercaptoethanol, 1 mm sodium orthovanadate, 40 mm β-glycerophosphate, 0.4 mm phenylmethanesulfonyl fluoride, 10% glycerol, 0.5% Triton X-100, and complete protease inhibitors). After brief sonication and protein concentration (determined by the BCA method), 30 μg of proteins were incubated in 100 μl of reaction mixture containing sphingosine (50 μM, delivered in 4 mg/ml fatty acid-free bovine serum albumin), [γ-32P]ATP (5 μCi, 1 mm dissolved in 10 mm MgCl2), and SK1 buffer for 30 min at 37 °C. The reaction was terminated by the addition of 10 μl of 1 n HCl and 400 μl of chloroform/methanol/HCl (100:200:1, v/v/v). Subsequently, 120 μl of chloroform and 120 μl of 2 m KCl were added, and samples were centrifuged at 3000 × g for 5 min. 200 μl of the organic phase was transferred to new glass tubes and dried. Samples were resuspended in chloroform/methanol/HCl (100:100:1, v/v/v). Lipids were then resolved on silica thin layer chromatography plates using 1-butanol/methanol/acetic acid/water (8:2:1.2, v/v/v/v) as solvent system and visualized by autoradiography. The radioactive spots corresponding to SIP1 were scraped from the plates and counted for radioactivity. Background values were determined in negative controls in which sphingosine was not added to the reaction mixture.

In Vivo Assay for Sphingosine 1-Phosphate Formation and Release—U87MG cells were seeded at 1 × 10^5 cells in 35-mm dishes and treated or not with siRNA for HIF-1α or HIF-2α. After 48 h, the medium was removed, and cells were serum-starved for 16 h and treated with CoCl2 for 2 h. At the end of incubation, cells were pulsed with 20 nm d-erythro-[3-3H] sphingosine (0.5 μCi/dish). Total lipids were extracted from cells at 4 °C with chloroform/methanol as previously reported (26, 47). Briefly, the total lipid extract was partitioned by high performance thin layer chromatography using n-butanol/acetic acid/water (3:1:1, v/v/v). Bands corresponding to the SIP1 standard were scraped, and the radioactivity was measured.
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using a scintillation counter. Extracellular S1P was extracted from pulse medium and partially purified as reported (26). The final organic phase containing S1P was submitted to high performance thin layer chromatography as above, and the S1P bands were scraped and counted as described above.

Western Blot Analysis—Western blot analysis was used to evaluate hSK1, HIF-1α, and HIF-2α. Cells were lysed in buffer containing 20 mM Hepes, pH 7.4, 50 mM NaCl, 1 mM EGTA, 0.5 mM β-glycerophosphate, 30 mM sodium pyrophosphate, 0.1 mM sodium orthovanadate, 1% Triton X-100, and protease inhibitor mixture tablet (Roche Applied Science) and kept on ice for 30 min. After brief sonication, protein concentration was determined using the BCA method, and 30 μg of protein was loaded on an SDS-10% polyacrylamide gel, transferred to a nitrocellulose membrane, and blotted with anti-hSK1, HIF-1α, HIF-2α, and actin. Immunoreactive bands were detected with horseradish peroxidase-conjugated anti-rabbit IgG (Jackson) and an ECL detection kit (Pierce).

In Vitro Tube Formation Assay—An in vitro tube formation assay was performed to evaluate the angiogenic properties of U87MG-conditioned medium on endothelial cells. HUVECs were seeded in 100-mm dishes at a concentration of 5 × 10^5 and used when they reached 80% confluence. At the day of the experiment the fluorescent cellTracker™ Green CMFDA (Molecular Probes) was added to the cells at 5 μM final concentration and incubated for 45 min. Medium was then removed, and cells were further incubated for 4 h in EGM-2 medium (without fetal bovine serum and singleQuots®) containing 0.1% fatty acid-free BSA. At the end of the incubation, cells were trypsinized and seeded at 4 × 10^4 cells/cm² in a 24-well plate precoated with growth factor-reduced Matrigel™ matrix (BD Biosciences) (450 μg/well of Matrigel diluted 1:1 with serum-free EGM-2) in DMEM, DMEM containing 100 nM S1P, or DMEM conditioned from U87 (incubated in 1 or 20% oxygen for 2 h) in the absence or presence of 1 μM VPC 23019. Tube formation was observed using a laser-scanning confocal microscope (LSM 510 Meta; Carl Zeiss, Thornwood, NY) after 16 h.

Cell Migration Assay—HUVECs were seeded in 100-mm dishes, loaded with the fluorescent cellTracker™ Green CMFDA, and serum-starved for 4 h as described above. After trypsinization, 1 × 10^5 cells were resuspended in 250 μl of EGM-2 medium containing 0.1% fatty acid-free BSA and placed in the upper chamber of a BD Falcon FluoroBlok™ 24-well plate precoated with 2 μg of rat tail collagen (Roche Applied Science). The lower compartment was filled with 750 μl of DMEM + 0.1% BSA in the absence or presence of different S1P concentrations or with conditioned medium from U87-MG in the absence or presence of 1 μM VPC 23019. After 4 h, the fluorescent migrating cells that had passed through the membrane to its lower surface were evaluated from the bottom in a fluorescent plate reader at excitation/emission wavelengths of 490/520 nm. Migratory ability was calculated as percentage of control (no chemottractant in the bottom well).

Other Methods—Radioactivity was measured by liquid scintillation counting. Human VEGF concentrations in cell culture medium were determined using a QuantiGlo chemiluminescent enzyme-linked immunosorbent assay (R&D Systems) according to the protocol provided by the manufacturer. S1P mass was quantified using the positive mode electrospray ionization/tandem mass spectrometry analysis in the Medical University of South Carolina Lipidomics Core Facility exactly as described (48). Total protein was assayed using the BCA method (Pierce) using bovine serum albumin as a standard. Cell viability was assessed by a trypan blue exclusion test, and a cell cytotoxicity test was performed using the cytotoxicity detection kit (lactate dehydrogenase) (CytoTox 96; Promega, Madison, WI). Statistical Analysis—All of the experiments were performed in triplicate. The results are presented as mean ± S.D. values or as representative results from at least three independent experiments. The statistical significance of the data was assessed with a two-tailed unpaired Student’s t test. A p value of <0.05 was considered statistically significant.

RESULTS

Effects of CoCl2 and Hypoxia on HIF-1α, HIF-2α, and SK1 Levels—To establish a model of hypoxia, CoCl2, a well known hypoxia mimic, was used to treat U87MG glioma cells, and their response was evaluated by determining levels of HIF-1α and HIF-2α. Fig. 1A demonstrates that CoCl2 (150 μM) treatment of serum-starved glioma U87MG cells caused an increase in both HIF-1α and HIF-2α protein levels in a time-dependent manner starting as early as 30 min of CoCl2 incubation.

Next, to determine if SK1 is up-regulated during hypoxic stress, U87MG glioma cells were treated with CoCl2, and cells were then harvested and assayed by Western blot analysis. As shown in Fig. 1A, SK1 protein levels increased during CoCl2 administration, starting at 1 h of treatment and reaching a maximal 2-fold increase after 2 h of treatment. In this cell line, our anti-hSK1 antibody recognizes two SK1-specific bands with similar molecular weights with identical kinetics of up-regulation, suggesting that there are two isoforms of SK1 that are up-regulated in response to CoCl2 treatment. Parallel experiments were conducted using a dual gas incubator (0.5% O2, 37 °C) to establish that hypoxia indeed regulates SK1 protein expression levels. As shown in Fig. 1B, after a 2-h exposure to low oxygen tension, HIF-1α and HIF-2α protein expression was increased, with a parallel increase in SK1 levels.

Next, to determine if the increase in SK1 protein level was due to an increase in the SK1 message, U87MG glioma cells were again treated with CoCl2 and harvested for real time PCR analysis. As shown in Fig. 1C, SK1 mRNA expression was also increased with a maximal 2-fold increase occurring after 4 h of CoCl2 treatment and subsequently returned to base-line expression levels after 16 h of CoCl2 treatment. Moreover, similar results on SK1 mRNA expression were obtained after hypoxia exposure (Fig. 1D). Of note is that SK2 mRNA levels remained unchanged in response to either hypoxia or CoCl2 (Fig. 1, D and C). These data suggest that, in glioma cells, SK1 is up-regulated when oxygen tension is drastically reduced, which could occur during rapid and abnormal tumor cell proliferation.

Effect of CoCl2 and Hypoxia on SK Activity and Intracellular and Extracellular S1P Production—SK1 is directly involved in the production of S1P, with activation of this enzyme influencing the levels of this important biomodulator within the cell. To
determine if the observed increase in SK1 protein expression also results in a higher level of S1P formation, we measured cellular SK activity. U87MG cells were serum-starved overnight and incubated with 150 μM CoCl2 for various times. As shown in Fig. 2A, SK1 activity significantly increased starting from 0.5 h of treatment (181.6 ± 18.9% compared with untreated controls), with maximal activation occurring after 1 h (192.1 ± 31.1%). SK1 activity was subsequently restored to basal levels after a 4-h treatment with CoCl2.

Next, we wanted to analyze if the increase in SK1 activity observed during CoCl2 treatment resulted in higher levels of intracellular and extracellular S1P. To achieve this, we used an "in vivo" assay of S1P formation, in which living cells were pulsed in culture with d-[erythro-3H]sphingosine. Specifically, U87MG cells were serum-starved for 16 h, treated for 2 h with 150 μM CoCl2, and then pulsed for 10 min with 20 nM [3H]sphingosine. As shown in Fig. 2B, U87MG produced higher amounts of S1P after CoCl2 stimulation, compared with untreated control cells. Moreover, analysis of the medium after the [3H]sphingosine pulse revealed that S1P is basally present in the extracellular milieu, but its levels were significantly increased after CoCl2 stimulation. In particular, the intra- and extracellular levels were increased by 21 and 59%, respectively. Importantly, the higher levels of extracellular S1P detected were not accompanied by LDH release, suggesting that S1P is actively released from intact cells and not a consequence of cell membrane rupture. To further support the hypothesis of S1P regulation during hypoxia, we incubated U87MG for 2 h in a 0.5% oxygen incubaor, and we analyzed the S1P mass levels. The results showed a significant increase of intracellular S1P under hypoxic conditions as compared with the normoxic conditions (0.498 ± 0.074 and 0.139 ± 0.02 pmol/mg proteins, respectively). Mass analysis, however, was not as sensitive as the [3H]sphingosine-based assay to detect extracellular S1P levels.

Effect of HIF-1α and HIF-2α Down-regulation on SK1 Expression—HIF-1α and HIF-2α regulate a battery of genes involved in the adaptive response of tumor cells to low oxygen availability. Therefore, we wanted to evaluate if SK1, which is up-regulated during acute hypoxia, is regulated by HIF-1α or...
HIF-2α in U87MG glioma cells. To address this, HIF-1α or HIF-2α were down-regulated using siRNA technology. Cells were treated with scrambled (sc in Fig. 3), HIF-1α, or HIF-2α siRNA for 48 h. After 16 h of serum starvation, cells were incubated in the absence (ct) or presence of CoCl2 for 2 h. As shown in Fig. 3D, both HIF-1α and HIF-2α were efficiently down-regulated by siRNA. Moreover, analysis of HIF-1α and HIF-2α mRNA levels demonstrated that both sequences were highly specific in their down-regulation of the genes of interest (data not shown). Interestingly, we observed a reciprocal relationship between the expression of HIF-1α and HIF-2α in U87MG glioma cells during hypoxia, such that HIF-1α siRNA amplified the hypoxic induction of HIF-2α protein levels and vice versa (Fig. 3D). A Western blot for SK1 expression revealed that SK1 induction during CoCl2 treatment was decreased with HIF-2α siRNA treatment. On the other hand, SK1 induction during CoCl2 treatment was enhanced in HIF-1α-silenced cells, which contained a higher level of HIF-2α (Fig. 3D). Similar results were obtained after SK1 mRNA analysis by real time PCR (Fig. 3A).

Next, the SK1 promoter sequence was examined and found to contain several putative HREs (supplemental Fig. 1). Therefore, to evaluate if the increase in SK1 was at the transcriptional level, a promoter construct with 3124 bp upstream of the SK1 transcription start site was subcloned in front of the luciferase gene. As shown in Fig. 3C, transient transfection of this construct into U87MG cells demonstrated a significant increase in luciferase activity by CoCl2 within 2 h of treatment (Fig. 3B), thus indicating that the SK1 response is at least in part due to transcriptional up-regulation of the SK1 message. Of note, SK1 promoter basal activity and induction during CoCl2 treatment decreased with HIF-2α siRNA treatment and increased with HIF-1α siRNA treatment, thus indicating that both of the transcription factors modulate SK1 promoter in normoxic and hypoxic conditions.

Next, to determine if the HREs in the SK1 promoter are functional, a ChIP assay was performed. As shown in Fig. 3C, ChIP analysis revealed that both HIF-1α and HIF-2α bind to the SK1 promoter under normoxic conditions. Moreover, after CoCl2 treatment, HIF-2α became more associated with the SK1 promoter, whereas HIF-1α showed a decrease in its SK1 promoter affinity (Fig. 3C). Importantly, the binding of both transcription factors appears to be specific to a region of the SK1 promoter (supplemental Fig. 1) containing one of the putative HREs, which is conserved in the human and rat genomes. In fact, only the pair of primers for this region showed an amplified PCR product after immunoprecipitation with HIF-1α and HIF-2α antibodies, whereas the other three pairs of primers did not show an amplified PCR product after immunoprecipitation (data not shown). Altogether, these data suggest that HIF-2α binds the SK1 promoter and is probably responsible for the increase in SK1 expression under hypoxia. On the other hand, HIF-1α appears to negatively regulate SK1 expression.

To further assess the contribution of HIF-2α in the regulation of SK1 levels, we used the renal carcinoma cell line 786-O, which is known to only express HIF-2α and not to express HIF-1α (49). For this purpose, we incubated HIF-2α or control siRNA-treated 786-O cells with 150 μM CoCl2 for 8 h. As shown in Fig. 4A, HIF-2α protein levels increased at 1 h of CoCl2 incubation, with a maximal increase after 4 h of treatment. Importantly, also in this cell model, SK1 protein was increased in a similar time-dependent fashion. Moreover, SK1 expression was reduced after HIF-2α down-regulation, indicating that HIF-2α is probably involved in SK1 up-regulation. It should be noted, however, that in 786-0 cells, HIF-2α is expressed constitutively in the absence of CoCl2, and consequently HIF-2α siRNA decreased basal SK1 levels. Moreover, ChIP experiments performed in 786-O confirmed that HIF-2α binds the SK1 promoter under normoxia and that this interaction increased dramatically under hypoxic conditions (Fig. 4B). These data lend...
A

150 μM CoCl₂

Time, h

0 1 2 4 8

siRNA
sc HIF-2α HIF-2α HIF-2α sc HIF-2α HIF-2α sc HIF-2α

SK1

β-actin

FIGURE 4. Effect of HIF-2α down-regulation on SK1 expression and ChIP analysis in O-786 cells. A, O-786 cells were treated with 50 nM scramble (sc) and HIF-2α siRNA for 48 h. Cells were then incubated in 150 μM CoCl₂ for different times and submitted to Western blot analysis for hSK1 (1:500), HIF-2α (1:1000), and β-actin. B, O-786 cells were incubated for 2 h in 150 μM CoCl₂, and the lysates were incubated with a nonspecific rabbit IgG (n.s.) and HIF-2α antibodies. Bands represent the PCR product obtained after immunoprecipitation (IP) using a specific pair of primers, called B (see supplemental Fig. 1). For all of the panels, a representative result from three independent experiments is shown.

further support to the notion that HIF-2α is involved in SK1 up-regulation during hypoxia.

Effect of HIF-1α and HIF-2α Down-regulation on Intracellular and Extracellular S1P in U87MG Glioma Cells—To gain further insights into the role of HIF-1α and HIF-2α in SK1 activation and S1P production, we determined the effect of HIF silencing on regulating intra- and extracellular S1P levels. As shown in Fig. 5A, on the other hand, down-regulation of HIF-1α resulted in increased intracellular S1P in both untreated and CoCl₂-treated cells. On the other hand, HIF-2α siRNA abrogated the increase in intracellular S1P observed after CoCl₂ administration. These results may indicate that HIF-1α is constitutively expressed in U87MG cells under normoxic conditions and may negatively regulate SK1 expression. Parallel results were obtained after analysis of extracellular S1P (Fig. 5B). These data suggest that both HIF-1α and HIF-2α are able to regulate SK1 protein level and activity, leading to the modulation of the intra- and extracellular levels of S1P.

Effect of Conditioned Medium from U87MG Glioma Cells after Hypoxia Treatment on HUVEC Migration and Tube Formation—Next, it was important to evaluate whether the S1P released into the extracellular compartment after hypoxic stress has a physiologic autocrine and/or paracrine function by its ability to regulate vascular formation in an in vitro model of human endothelial cellular vessel formation in Matrigel. Using this assay, we first show that serum-free medium when added to endothelial cellular vessel formation in Matrigel. Using this

FIGURE 5. Effect of HIF-1α and HIF-2α down-regulation on intracellular and extracellular S1P in U87MG glioma cells. U87MG cells were treated with 50 nM scramble (sc), HIF-1α, or HIF-2α siRNA for 48 h and incubated with 150 μM CoCl₂ for 2 h. Cells were then pulsed with 20 nM of [3H]sphingosine and further incubated for 10 min. Cells and media (A and B, respectively) were then collected and analyzed for the radioactive sphingosine 1-phosphate content. The data shown are the means ± S.D. from three experiments performed in duplicate. *, statistically significant difference (p < 0.05) compared with control (scrambled in DMEM + BSA).

FIGURE 6. Hypoxia Treatment on HUVEC Migration and Tube Formation

HUVECs as expected did not induce noticeable tube formation assay, we first show that serum-free medium when added to endothelial cellular vessel formation in Matrigel. Using this

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FIGURE 6. Hypoxia Treatment on HUVEC Migration and Tube Formation

HUVECs as expected did not induce noticeable tube formation assay, we first show that serum-free medium when added to endothelial cellular vessel formation in Matrigel. Using this

regulate vascular formation in an physiologic autocrine and/or paracrine function by its ability to

into the extracellular compartment after hypoxic stress has a

Next, it was important to evaluate whether the S1P released

to regulate SK1 protein level and activity, leading to the modu-

lations and may negatively regulate SK1 expression. Parallel

results were obtained after analysis of extracellular S1P (Fig.

B). These data suggest that both HIF-1α and HIF-2α are able to

regulate SK1 protein level and activity, leading to the modu-

lation of the intra- and extracellular levels of S1P.
To quantitate the effect of hypoxia-conditioned medium on endothelial cell function, a HUVEC migration assay was performed. HUVECs were tested for migration in response to conditioned medium using a modified Boyden chamber transwell system. As expected (Fig. 6B), S1P was a very potent chemoattractant molecule and caused a 2-fold increase in cell migration with different concentrations of S1P (0.3 μM to 300 μM) or filled with DMEM containing 0.1% BSA (B) for 48 h and then incubated for an additional 16 h with conditioned medium obtained from U87MG cells incubated for 2 h in 0.5% oxygen. These data suggest that increased S1P levels in the medium from U87MG glioma cells after brief exposure to hypoxia may be sufficient to induce HUVEC angiogenic responses (migration and tube formation) through the activation of S1P receptors on endothelial cells.

**DISCUSSION**

SK activity and S1P production are essential for complete vasculogenesis and angiogenesis during embryonic development. In addition, S1P can act as an angiogenic factor in several models of malignancy (2–4). Although hypoxia has been linked to an up-regulation of VEGF and promotion of angiogenesis, no clear link between SK activation and S1P production in hypoxia has been established to date. Therefore, in this study, we wished to investigate the role of SK1 in the tumor hypoxia response. Using a cell line derived from human glioblastoma, a tumor type that, in vivo, is characterized by extensive areas of hypoxia and a prominent angiogenic response, we sought to determine if SK activation and S1P production are altered during hypoxia. The results demonstrate for the first time that SK1, the enzyme directly responsible for S1P production, is up-regulated during tumor cell hypoxia and that HIF-2α, but not HIF-1α, is involved in the up-regulation of SK1 under hypoxic conditions. Interestingly, we observed that these two transcription factors, which are both dramatically increased during tumor hypoxia in glioma cells, are reciprocally regulated and have opposing effects on SK1 expression. Moreover, we show that the increasing SK expression in response to hypoxia is sufficient to increase not only intracellular S1P levels but also extracellular S1P, the latter of which can act as a potent angiogenic stimulus for endothelial cells through its interaction with S1P-specific receptors.

The mechanism of SK1 regulation by hypoxia may be in part through its transcriptional up-regulation. In fact, analysis of the putative promoter region of hSK1 revealed several potential hypoxia-responsive elements, which are conserved motifs containing the consensus core sequence 5′- (A/G)CGT(G/C)(G/C)-3′ localized at varying positions and orientations of the coding regions of several hypoxia-regulated genes (51). Indeed, we found that, in U87MG glioma cells, CoCl2 and hypoxia increased SK1 message and protein levels. In addition, we demonstrated that SK1 promoter construct is activated by CoCl2 and HIF-1α and HIF-2α bind the SK1 promoter in a hypoxia-regulated manner.

Up-regulation of SK1 expression in hypoxia may have important implications to tumor progression. A commonly shared feature of solid cancers is the presence of regions with low vascular perfusion, often called “hypoxic regions,” which occur as a result of abnormal and rapid cellular proliferation not accompanied by an adequate growth in their vascular networks. It has been shown that in turn hypoxic environments enhance a process labeled the “angiogenic switch” in tumor cells, a process largely characterized by a transcriptional response. This transcriptional response leads to the release of proangiogenic factors, such as VEGF (52), and a decline in antiangiogenic factors, leading to neovessel formation. Our results show that after 2 h of hypoxia, there was an increase of released S1P but not of VEGF in the medium, suggesting that SK1 activation and S1P
release take place before VEGF activation and release. Initiation of the angiogenic switch, with subsequent vascularization, is one of the major rate-limiting steps in the growth of solid tumors. It appears very likely that SK1 up-regulation may be part of this angiogenic switch. Indeed, compelling evidence from the literature suggests that SK1 mRNA is up-regulated in many types of solid tumors (30, 34, 53). Moreover, elevated levels of SK1 protein were also reported in human non-small cell lung carcinoma when compared with normal adjacent tissue (30). Similar findings were also reported in murine colon adenocarcinomas in an azoxymethane colitis model of colon cancer (31). Our data showing for the first time that SK1 is up-regulated during hypoxia allow us to speculate that this activation could be an explanation for the higher level of SK1 observed in solid tumors.

The finding that SK1 up-regulation by hypoxia is accompanied by a functional increase in S1P production and release into the extracellular space also has important implications to tumor biology. S1P is emerging as a new and highly potent proangiogenic factor, which acts on endothelial cells through activation of specific G-coupled receptors S1P1-5 (5). In particular, it has been demonstrated that S1P is sufficient for the induction of HUVEC tube formation within a Matrigel matrix in vitro but also in vivo as assessed by a Matrigel plug assay (54). Interestingly, a recent study reported that the presence of an anti-S1P antibody into several mice xenograft models is able to inhibit in vivo angiogenesis and tumor growth (55). In our system, we observed a potent effect of conditioned medium obtained from hypoxia-treated U87MG cells on in vitro endothelial tube formation and cell migration. This effect was in part mediated by S1P, since HUVECs pretreated with VPC23019, a selective inhibitor of S1P1, or HUVECs in which S1P1 has been silenced by siRNA lost the ability to form tubes and migrate in the presence of U87MG-conditioned medium. It is therefore interesting to propose a model whereby, upon sensing hypoxia, tumor cells increase SK1 levels and activity, leading to release of S1P that in turn results in de novo blood vessel formation (Fig. 7).

The angiogenic switch in a hypoxic environment has been largely attributed to another important proangiogenic growth factor, VEGF, which is transcriptionally activated and secreted in response to tumor hypoxia (60). Interestingly, it has been demonstrated that VEGF and S1P can act synergistically to form new vessels. Substantial cross-talk exists between S1P and VEGF, such that S1P transactivates VEGF-2 receptor (56) and VEGF up-regulates both S1P3 (57) and S1P1 receptor expression associated with an enhanced intracellular signaling response to S1P (58). Although in this paper, we did not investigate a possible transactivation between S1P and VEGF during tumor hypoxia in glioma tumor, it will be interesting to determine if an increase in S1P production leads to a concomitant increase in VEGF production or efficacy, as suggested by recent studies (55).

An unexpected observation from this study is that the transcription factor HIF-2α, but not HIF-1α, is involved in the up-regulation of the SK1 gene. In fact, after down-regulation of HIF-2α, hypoxia no longer led to an increase in SK1 mRNA, protein, or S1P production and release in glioma cells U87MG. Moreover, a promoter activity assay demonstrates that the SK1 promoter is activated by CoCl2 in a HIF-2α-dependent manner. Notably, as demonstrated by this assay, HIF-2α is also an important regulator of the basal SK1 activity in cells, such that down-regulation of HIF-2α resulted in lower SK1 promoter activity under normoxic conditions. Importantly, as demonstrated by ChIP analysis, both of the transcription factors directly bind the putative SK1 promoter under normoxia, but HIF-2α association increases and HIF-1α association decreases under hypoxia. Our hypothesis is that the two transcription factors may compete for SK1 promoter binding during normoxia or hypoxia, leading to different SK1 expression. Similarly, HIF-2α directly binds to the SK1 promoter in O-786 renal adenocarcinoma cells, which lack HIF-1α (57). Moreover, HIF-2α down-regulation in these cells also led to down-regulation of SK1 expression. Importantly, down-regulation of HIF-1α increased SK1 levels and S1P production and release. It is noteworthy that among the two HIF transcription factors, HIF-1α is the better characterized. Significantly less is known about the role HIF-2α plays in hypoxic responses or what biological functions it may serve. Recent evidence in the literature, reported by Raval and colleagues (59), supports a distinct role for HIF-1α and HIF-2α in renal cell carcinoma. They showed that in these tumors, HIF-1α and HIF-2α, despite close structural similarities, have contrasting functions, with HIF-1α retarding and HIF-2α enhancing tumor growth. In particular, they found that HIF-1α positively regulates apoptosis-regulated proteins and activates caspase-independent necrosis-like cell death (60) with no effect on cell cycle progression or VEGF production. In contrast, they showed that HIF-2α negatively regulates proteins involved in the apoptotic pathway and positively regulates entry into the cell cycle and enhancing VEGF production and release. It is therefore not surprising that SK1 is differentially regulated by HIF-1α and HIF-2α, since down-regulation of SK1 by HIF-1α could be necessary for cells to undergo apoptosis, and its up-regulation by HIF-2α could be needed for its proangiogenic activity. Cellular responses to hypoxia via differential regulation of SK1 may be one way by which cancer cells could achieve a balance between growth and the need for blood vessel supply. In fact, a specific role of HIF-2α in glioma tumor biology came from Khatau et al. (61), who demonstrated that overexpression of HIF-2α but not HIF-1α occurs in high grade astrocytoma. In particular, they found that unlike HIF-1α, which was found to be similarly expressed in both high grade astrocytomas and low grade astro-
cytomas, HIF-2α was preferentially overexpressed in high grade astrocytoma. Moreover, it has been shown that overexpression of HIF-2α was associated with high vascular grade and poor survival in breast carcinomas (62). These observations, together with our data, lend support for a specific role of HIF-2α in tumor angiogenesis. We propose that a possible advantage of HIF-2α-overexpressing cancer cells could in part depend on their ability to up-regulate SK1 and increase S1P formation to promote angiogenesis.

In summary, our data indicate for the first time that HIF-1α and HIF-2α have functionally distinct roles in the regulation of SK1 and S1P levels, with HIF-1α inhibiting and HIF-2α activating SK1 expression during tumor hypoxia. This discovery may have important implications to understanding glioma tumor biology during hypoxia. More importantly, our research implicates a link between S1P production as a potent angiogenic agent and the hypoxic phenotype observed in many tumors. Targeting specific HIF pathways could be an effective strategy to decrease S1P production and release to inhibit glioma tumor growth by decreasing angiogenesis.

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